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Biotransformation of hydroxychloroquine to evaluate the cytotoxicity of its metabolites and mimic mammalian metabolism

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ABSTRACT

Hydroxychloroquine (HCQ) displays attractive anti-inflammatory and antiviral effects. Because of that, such a drug made part of some clinical trials for combating Sars-CoV-2 during the COVID-19 pandemic. The present study aimed to conduct the biotransformation of HCQ by filamentous fungi reported as microbial models of mammalian drug metabolism to evaluate its cytotoxic after metabolization. *Cunninghamella echinulata* var. *elegans* ATCC 8688a could efficiently biotransform HCQ into one main metabolite identified as the new 4-(1,2,3,4-tetrahydroquinolin-4-ylamino)pentan-1-ol (HCQ-M). The microbial transformation occurred through *N*-dealkylation, 7-chloro-elimination, and reduction of the two conjugated double-bond from the quinoline system of HCQ. The cytotoxic profiles of HCQ and its metabolite were evaluated using CCD-1059Sk cells (human fibroblasts) through sulforhodamine B, trypan blue, and Live/Dead assays. Both HCQ and HCQ-M displayed cytotoxic activities in human fibroblasts, but HCQ-M was significantly more toxic than HCQ. The reported findings should be considered for further clinical studies of HCQ and will be important for guidance in achieving new derivatives from it.

Introduction

Chloroquine has been extensively used as an antimalarial drug [27]. Its hydroxy-derivative, the hydroxychloroquine sulfate (HCQ), was first synthesized in 1946 and was demonstrated to be much less (~40 %) toxic than chloroquine in animals [14]. HCQ displays attractive anti-inflammatory action, which explains its clinical use for rheumatic disease treatment [3,7] and antiviral effects [17]. The antiviral activity of HCQ was a point of interest to different worldwide researchers to prove its effectiveness in inhibiting the novel coronavirus (responsible for the COVID-19 pandemic), and some *in vitro* trial registries have been published [13,26].

HCQ is clinically administered as a racemate; it is well absorbed (70–80 % bioavailability), and between 21 and 47 % of the dose is excreted unchanged [19]. HCQ is generally considered safe. Its most common side effects include gastrointestinal symptoms, and severe side effects have a low incidence, which include neuromyopathy of proximal muscles, cardiotoxicity, skin hyperpigmentation, and irreversible retinopathy [23]. Due to the interesting biological effects of HCQ, it is mandatory to comprehensively know about possible toxic effects resulting from its metabolization.

After human oral administration, drugs suffer several metabolic reactions by the action of a wide array of body enzymes. In humans, the metabolism of xenobiotics may result in metabolic bioactivation producing toxic chemical species, and their involvement in causing adverse drug reactions has been extensively studied and reported [15]. The costs

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and ethical concerns sometimes hinder the study of human metabolic responses, the characterization of the drug metabolites, and evaluating of their cytotoxicity.

Microbial biotransformation approaches have been applied as part of preclinical screening for new drug candidates [6] since they may provide information about the safety of any drug through knowledge about the metabolism [1]. Microorganisms are used as models for mammalian drug metabolism to establish biologically active compounds' metabolic fate and provide sufficient amounts of metabolites for structure elucidation and biological evaluation [8]. The use of microbial models for simulating the mammalian metabolism of bioactive compounds is well established in the literature, and our research group has been dedicated to this topic [16,22,21]. That approach contributes to a better understanding of human drug metabolism. It provides a simplified version of the complex human metabolic pathways, allowing the isolation of metabolites in enough quantity for their chemical identification and toxicity evaluations.

Chemical modifications by enzymes have been studied for hundreds of years [4] and enhanced with the publication of the Food and Drug Administration (FDA) recommendation (1992) regard to the use of only one enantiomer of the chiral bioactive agents [5]. That is why the chemical transformations enzyme-catalyzed have raised as attractive tools to achieve new derivatives of drugs under regio- and stereocontrols.

Fungi are the most studied catalysts for *in vitro* metabolic studies [2] since they may metabolize a wide diversity of compounds, leading to chemical derivatives through selective reactions that work under ecofriendly conditions. An impressive number of filamentous fungi strains have been shown to possess broad catalytic properties on different substrates. Mainly, the *Cunninghamella* genus is extensively used as a microbial model for mammalian hepatic metabolism of several compounds. It is stated that *Cunninghamella* sp. can metabolize several drugs through reactions similar to those catalyzed by mammalian enzyme systems [12]. Due to ethics and safety issues, fungi are excellent alternatives to *in vivo* human metabolism studies.

Although the correlation between reactions catalyzed by microorganisms and human metabolisms is not trivial, microbial biotransformation has been helpful for *in vitro* drug metabolism studies by providing information and standards for *in vivo* studies. The present study aimed to conduct the *in vitro* transformation of HCQ by filamentous fungi reported as microbial models of mammalian drug metabolism to evaluate its cytotoxicity after metabolization. We are reporting a new derivative of HCQ, which displayed more cytotoxicity towards human fibroblasts than the HCQ. The herein-developed approach highlights the importance of comprehensive knowledge of the metabolism of drugs to assess their toxicity.

Material and methods

General experimental procedures

All chemicals and solvents were purchased from commercial suppliers and used without further purification. ^{1}H NMR spectrum was recorded at 400 MHz, with a Bruker Avance III HD spectrometer (Bruker, Billerica, USA). Chemical shifts (δ) were referenced to the residual deuterated methanol (CD_3OD) peak at δ_H 3.31 for ^{1}H.

The high-performance liquid chromatography – diode array detector (HPLC-DAD) analyses were performed using a C12 column (Phenomenex 250 mm × 4.6 mm × 5 μm) and gradient elution system composed of 5 to 100 % methanol (HPLC grade Tedia, Rio de Janeiro, Brazil) in water over 30 min at a flow rate of 0.5 mLmin^{-1}. The crude extracts were analyzed by injection of 10 μL at mgmL^{-1} on a Shimadzu (SIL-20A) multi-solvent delivery system (Shimadzu SPD-M20A) equipped with an SPD-M20A photodiode array detector (DAD) and an Intel Celeron computer for analytical system control, data collection, and processing. The chromatographic profiles were recorded at λ 343 nm.

The ultra-high performance liquid chromatography - high-resolution mass (UHPLC-HRMS) analyses were carried out at a spectrometer operated at both positive and negative modes, using a C18 column (ACE 150 mm × 4.6 mm × 3 μm). UHPLC-HRMS contained an electrospray ionization (ESI) source and an Orbitrap technology analyzer. The flow rate was 400 μL.min^{-1}, and the analytical method was the same as that used in HPLC-DAD analyses. The column temperature was controlled at 30 °C. The scanning range of 120–1200 *m/z* to full MS; ESI MS resolution 70.000 with lock mass; microbeam, 1; and maximum injection time, 250 ms. The parameters of the ESI ionization source were as follows: gas flow rate, 30; auxiliary gas flow rate, 10; positive voltage spray mode, 3.6 kV; negative voltage spray mode, 3.2 kV; and Slens level = 55. Nitrogen gas was used as a nebulizer. The clean-up of the samples was performed before the analysis with hexane (2 × 200 μL) to ensure the removal of nonpolar substances and filtered using an HPLC PTFE syringe filter of pore size 0.45 μm. The mass spectra were obtained and processed using Xcalibur (Thermo Fisher Scientific).

Substrate

Hydroxychloroquine sulfate was purchased from Sigma-Aldrich (purity 98 %).

Biotransformation and purification assays

The biotransformation of HCQ was done through screening with *Aspergillus brasiliensis* ATCC 16404, *Cunninghamella echinulata* var. *elegans* ATCC 8688a, and *C. elegans* ATCC 10028b, which were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The filamentous fungi were maintained in 80 % glycerol solution at −20 °C. The fungi were grown in a two-step culture procedure. First, each fungus was grown at 28 °C in Petri dishes containing malt agar (malt extract 2.0 %, glucose 2.0 %, peptone 0.1 %, agar 1.8 %) for 7 days. Next, an inoculum of ten 6-mm disks containing mycelia and agar was added to 250-mL Erlenmeyer flasks, each holding 100 mL of Koch's K1 medium (glucose 0.18 %, peptone 0.06 %, and yeast extract 0.04 %). HCQ (10 mg) was added to each flask as a solution in water. Control flasks consisted of a culture medium with fungus (without HCQ), a culture medium with HCQ (without a fungus), and a culture medium by itself. Biotransformation experiments were carried out at 28 °C for eight days with shaking at 120 rpm. Samples were analyzed daily by HPLC. The mycelia were separated by filtration, the fermentation broths were extracted three times with ethyl acetate, and the solvent was evaporated under reduced pressure to yield crude extracts. All experiments were carried out in triplicate. Using the same procedures, the biotransformation of HCQ by *C. echinulata* var. *elegans* was carried out separately in 10 Erlenmeyer flasks (scale-up biotransformations). The extraction of the culture broths by ethyl acetate was followed by evaporation of the solvent to yield the crude extracts of the biotransformations of HCQ (41.0 mg).

The main metabolite of HCQ (HCQ-M) was purified on a chromatographic column (40 × 1.5 cm) containing silica gel 60 Å (Sigma-Aldrich). The mobile phase consisted of gradients composed of *n*-hexane, ethyl acetate, and methanol. A total of 36 fractions were collected and fourth fraction contained the HCQ-M.

4-(1,2,3,4-tetrahydroquinolin-4-ylamino)pentan-1-ol: ^{1}H NMR (400 MHz, CD_3OD) δ 7.51 (2H, *d, J* = 8.0 Hz, H-5 and H-7), 6.84 (2H, *d, J* = 8.0 Hz, H-6 and H-8), 3.83–3.77 (3H, *m*, H-1′a, H1′b, and H-4), 3.12–2.97 (2H, *m*, H-2a and H-2b), 2.85–2.80 (1H, *m*, H-4′), 2.12–2.09 (2H, *m*, H-3a and H-3b), 1.32–1.24 (4H, m, H-2′and H-3′), 1.15 (3H, *d, J* = 8.0 Hz, H-5′); HRESIMS *m/z* 235.18091 $[M + H]^+$ (calcd for $[M + H]^+$ 235.18049).

Cytotoxic assay

CCD-1059Sk cells (fibroblasts derived from normal human skin)

were grown in a DMEM (Dulbecco's Modified Eagle Medium) containing sodium bicarbonate 5 mM and fetal bovine serum (FBS) 10 % at 37 °C in a 5 % CO_2. The culture media were renewed every-two days, and the cultures were subcultured at approximately 80 % confluence.

The following experiments were conducted in triplicate. The data shown are mean ± standard error of triplicates' mean (SEM). Significant differences from the control group (untreated cells) were determined according to the analysis of variance (ANOVA) followed by Tukey's test, and significant differences between the HCQ and HCQ-M groups were determined according to the ANOVA followed by Bonferroni's post-test: $p < 0.05$.

Cell viability assay

Cell viability was evaluated using the colorimetric sulforhodamine B (SRB) assay. This assay is based on the ability of SRB to bind protein components of cells fixed by trichloroacetic acid (TCA) in culture plates. SRB is a bright pink aminoxanthene dye with two sulfonic groups that attach to basic amino acid residues under moderately acidic conditions and dissociate under basic conditions. SRB binding is stoichiometric; therefore, the amount of dye extracted from stained cells is directly proportional to the number of viable cells [25].

For cell viability analysis using the SRB, cells were plated in 96-well plates at a density of 5×10^3 cells per well. After 24 h of plating, hydroxychloroquine (HCQ) or its metabolite (HCQ-M) was added at concentrations of 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0, and 400.0 μg/mL. The plates were incubated for 48 h. After the incubation, the cells were fixed with TCA 15 % for 1 h at 4 °C, then washed with MiliQ water and dried at room temperature. Each dry plate was revealed using 100 μL of 0.4 % SRB for 1 h at room temperature, protected from light. Then, an additional washing was performed using acetic acid 1 % to remove all SRB residue. After that, Tris Base 10 mM (pH 10.5) was added to solubilize the SRB for 30 min. Then, the optical density was measured in a spectrophotometer (Asys UVM 340) at 540 nm (background subtraction at 690 nm). Cell viability was expressed as percentages compared to control (untreated cells). IC_{50} values were determined from a non-linear regression using GraphPad Prism®.

Trypan blue assay

Trypan blue is a dye that penetrates into cells with damaged membrane [24].

For the trypan blue assay, cells were counted and plated in 24-well plates at a density of 2×10^4 cells per well in 400 μL of DMEM. After 24 h, the cells were treated with HCQ and HCQ-M at 50, 100, and 150 μg/mL concentrations for 24 and 48 h. After the treatments end, the cells were trypsinized and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended in a DMEM medium, and 20 μL of the cell suspension was mixed with 20 μL of trypan blue solution 0.4 % for 2 min. The cells and trypan blue solutions were placed in a Neubauer chamber, and the viable (unstained) and nonviable (blue stained) cells were counted with the aid of the optical microscope.

Live/Dead assay

The calcein-AM and propidium iodide (PI) assay evaluated cell viability (live) and apoptosis (dead). For that, cells were exposed to calcein acetoxymethyl ester (calcein-AM) that becomes fluorescent after cleavage of calcein-AM by cellular esterases, which produce a green fluorescent calcein derivative [12]. Cells in late apoptosis have their membrane permeable to PI due to the emergence of pores, which are red-stained [20].

For the calcein-AM/PI assay, cells were counted and plated in 35 mm plates containing a coverslip at a density of 8×10^4 cells per well in 2 mL of DMEM medium. After 24 h, the cells were treated with HCQ and HCQ-M at 50, 100, and 150 μg/mL concentrations for 24 and 48 h at 37 °C in a 5 % CO_2.

After the treatment, all medium was removed, and the wells were washed with PBS. Subsequently, a solution containing PI 5 μg/mL and calcein-AM 3 μM was added. The plates were incubated for 30 min at room temperature. After incubation, cells were observed under a fluorescence microscope at 20x magnification. Cells were counted in three different regions using Image J software (National Institutes of Health, USA).

Results and discussion

The biotransformation of hydroxychloroquine (HCQ) was screened with *Aspergillus brasiliensis* ATCC 16404, *Cunninghamella echinulata* var. *elegans* ATCC 8688a, and *Cunninghamella elegans* ATCC 10028b. Comparative HPLC-DAD analysis of the chemical profiles of extracts from biotransformation and control experiments showed that *C. echinulata* var. *elegans* was an efficient model for biotransformation of HCQ (Fig. 1). Analysis of chromatogram B of Fig. 1 showed that the fungus completely consumed the substrate with the production of some metabolites. The main metabolite was detected at a retention time of 13.0 min.

The products of any xenobiotic metabolism may be inactive metabolites with no pharmacological activity, named detoxification products; active metabolites which may have pharmacological properties of less, equal, or greater magnitude than their parent compounds; or reactive metabolites that are capable of covalently reacting with the functional macromolecular structure of endogenous targets *in vivo*, resulting in undesired toxic effects [15]. Our investigation of the biotransformation of HCQ had as its primary goal the assessment of possible toxicities displayed by its metabolites. For this, an initial screening of HCQ biotransformation was made using three fungi reported as microbial models of mammalian drug metabolism. Among the evaluated fungi, only *C. echinulata* var. *elegans* showed the ability to transform HCQ into less polar metabolites after four days of incubation (Fig. 1B).

It is well known that fungal species often contain more than a hundred cytochrome P450 genes [10]. Such microorganisms offer great potential to develop model systems to mimic the metabolism of drugs in humans, which is helpful for *in vitro* structure–activity relationship studies. Besides that, biotransformation by fungi is an interesting approach for achieving chemical derivatives from bioactive start materials.

The main microbial metabolite of HCQ by *C. echinulata* var. *elegans* (HCQ-M) was isolated by column chromatographic. The high-resolution ESI (*m/z* 235.18091 $[M + H]^+$, Fig. 2A) mass spectrum of HCQ-M indicated a molecular formula of $C_{14}H_{22}N_2O$, that was supported by MS^2 spectrum (Fig. 2B) analysis.

Additionally, a detailed analysis of the MS^2 spectrum (Fig. 2B) of HCQ-M and the proposition of some ion fragments (Fig. 4) confirmed its chemical structure. An intramolecular attack on the cationized structure of HCQ-M, followed by water loss, led to the cyclization of the side chain and the formation of an unstable ion. At the sequence, loss of C_9H_9N produced to fragment (*m/z* 86.09712) was seen in the MS^2 spectrum of HCQ-M. Finally, the ^1H NMR analysis confirmed the chemical structure of HCQ-M. Several signals appeared overlapping in the NMR spectrum and some multiples have been observed. However, the NMR analysis supported the proposition of the chemical structure of HCQ-M.

Biotransformation by microorganisms may simulating the mammalian metabolism and are part of the pre-clinical screening of new drug candidates [9]. The *N*-dealkylation forms the major metabolites of chloroquine and hydroxychloroquine in humans. Following administration, those compounds are rapidly *N*-dealkylated to the pharmacologically active desethylchloroquine and bis-desethylchloroquine by P450 enzymes [19]. UHPLC-HRMS analysis of HCQ-M was a fast and efficient technique for its chemical structure determination. ESI-MS and MS^2 data analysis showed that the microbial biotransformation catalyzed some interesting modifications in the HCQ scaffold resulting in the structure of its main metabolite (Fig. 3), which has zero hit on the comprehensive mass database. In accordance with studies about the metabolism of HCQ in humans, *C. echinulata* var. *elegans* also catalyzed

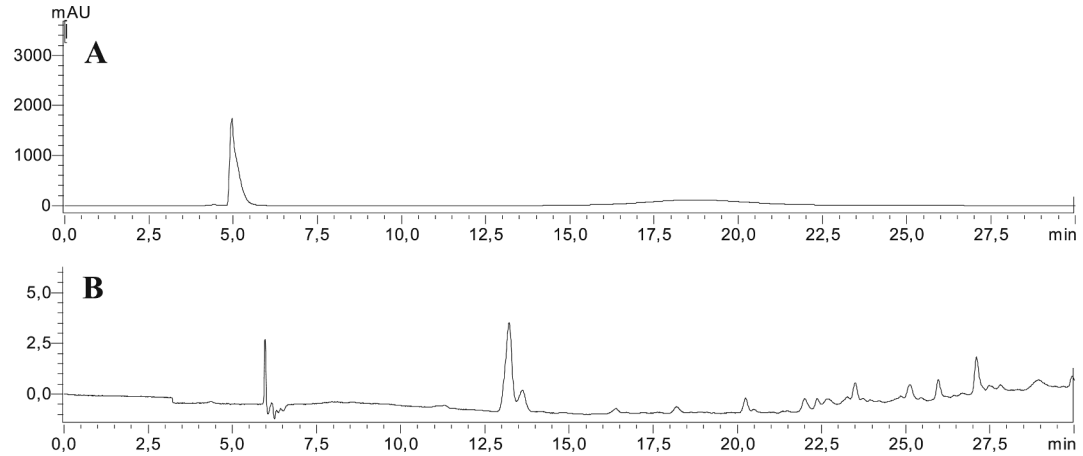

Fig. 1. HPLC-DAD elution profiles (λ = 343 nm) of hydroxychloroquine (**A**) and ethyl acetate extract from *C. echinulata* var. *elegans* ATCC 8688a cultures with hydroxychloroquine during 4 days (**B**). The main metabolite from hydroxychloroquine was visualized at chromatogram B (retention time 13.0 min). AU absorbance units.

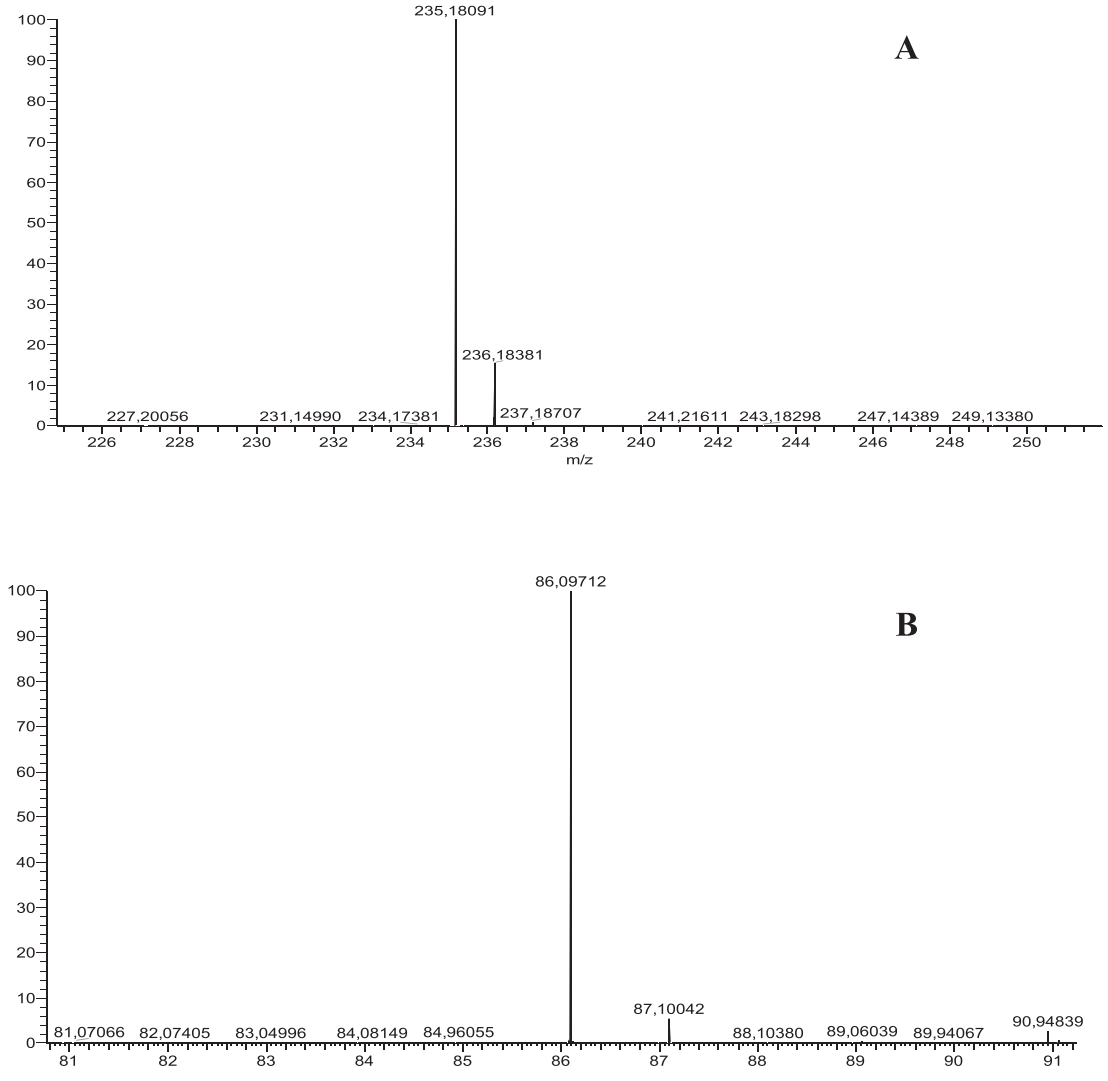

Fig. 2. ESI-Mass spectra (MS, **A**; and MS^2, **B**) of the microbial metabolite of hydroxychloroquine (HCQ-M) achieved through ultra-high performance liquid chromatography - high-resolution mass (UHPLC-HRMS) analysis.

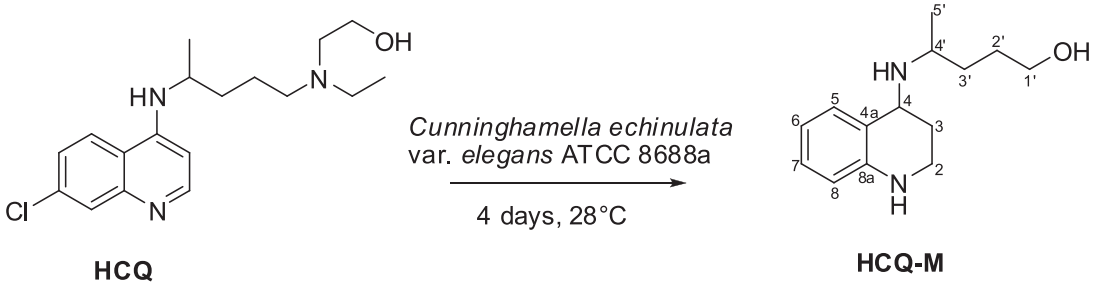

Fig. 3. Representation of biotransformation of hydroxychloroquine (HCQ) into its main metabolite (HCQ-M) by *Cunninghamella echinulata* var. *elegans* ATCC 8688a.

an *N*-dealkylation at the side chain of HCQ. Additionally, 7-chloro-elimination and reduction of the two conjugated double-bond from the quinoline system generated the chemical structure of HCQ-M.

It is stated that the elucidation of the metabolic pathway of any compound and the knowledge about the safety of its metabolites are critical points in drug development programs. Within this context, cell cytotoxicity assays were carried out with HCQ and HCQ-M on CCD-1059Sk cells. Cell viability was evaluated using the SRB assay showing that HCQ and HCQ-M displayed cytotoxicity towards CCD-1059Sk cells compared to control results (Fig. 5A). We found that HCQ-M was more cytotoxic than HCQ. IC_{50} values of HCQ and HCQ-M were 103.17 ± 5.29 μg/mL and 84.35 ± 0.89 μg/mL, respectivelly. Assays performed for 48 h showed a significant difference in CCD-1059Sk cell's viabilities at 200 μg/mL, with higher toxicity displayed by HCQ-M.

Trypan Blue and Live/Dead assays (Fig. 5B) corroborated the results and showed that the number of viable cells decreased at all tested concentrations of HCQ and HCQ-M compared to control after 24 h. Interestingly, HCQ-M also displayed more accentuated toxicity than HCQ and caused a significant decrease in the viability of the CCD-1059Sk cells at the three evaluated concentrations (50, 100, and 150 μg/mL).

Finally, cell viability and apoptosis were evaluated in CCD-1059Sk cultures treated with HCQ and HCQ-M through Calcein-AM and PI staining, respectively. Fig. 5C shows that both HCQ and HCQ-M displayed toxicity against CCD-1059Sk cells at all evaluated concentrations. HCQ-M increased apoptosis, and a significant difference between results with HCQ and HCQ-M was observed at all tested concentrations. Thus, the results suggest that the enhancement of apoptosis is one of the mechanisms associated with HCQ-M toxicity. It has already been demonstrated that HCQ treatment in fibroblast-like synoviocytes led to dose-dependent apoptosis through caspase-3 activation, which is a critical step for cell fragmentation and apoptotic cell death [11].

The SARS-CoV-2 pandemic and the global public health challenge led the necessity to find treatments urgently. HCQ gained worldwide attention, and, despite its established use as an antimalarial drug and in autoimmune disease treatment, its safety is still controversial. A recent study demonstrated that HCQ displayed cytotoxicity against several human cell lines and human lung fibroblast (IMR-90) (Yang et al., 2020). The cytotoxicity was time-dependent, suggesting that it is appropriate short period administration. HCQ cytotoxicity for human dermal fibroblasts has also been demonstrated, which supported the rationale for HCQ application as an antimalarial drug and treatment of human fibrosclerotic diseases [18]. Despite the relative safety of HCQ, to the best of our knowledge, we observed for the first time that the metabolite derived from HCQ (HCQ-M) was more cytotoxic than the HCQ.

Even at the minor evaluated concentration (50 μg/mL) of HCQ and HCQ-M, there was an alteration in the morphology of the cells (Fig. 6). Notably, the cells treated with HCQ or HCQ-M 50 μg/mL became larger than those from the control. In addition, several vacuoles appeared in the treated cells. Thus, it is worth mentioning that both HCQ and HCQ-M are toxic to cells at concentrations higher than 50 μg/mL. This cell morphology suggests that HCQ and HCQ-M at 50 μg/mL induce a type of cell death known autophagy.

The cytoplasmic vacuolization of human dermal fibroblasts and HaCaT under light microscopy was observed after the treatment with HCQ. Besides these autophagic vacuoles, it was further demonstrated that HCQ induces the autophagic regulators LC3B-II and Beclin-1 at both RNA and protein levels confirming that HCQ induced autophagy in these cells [18].

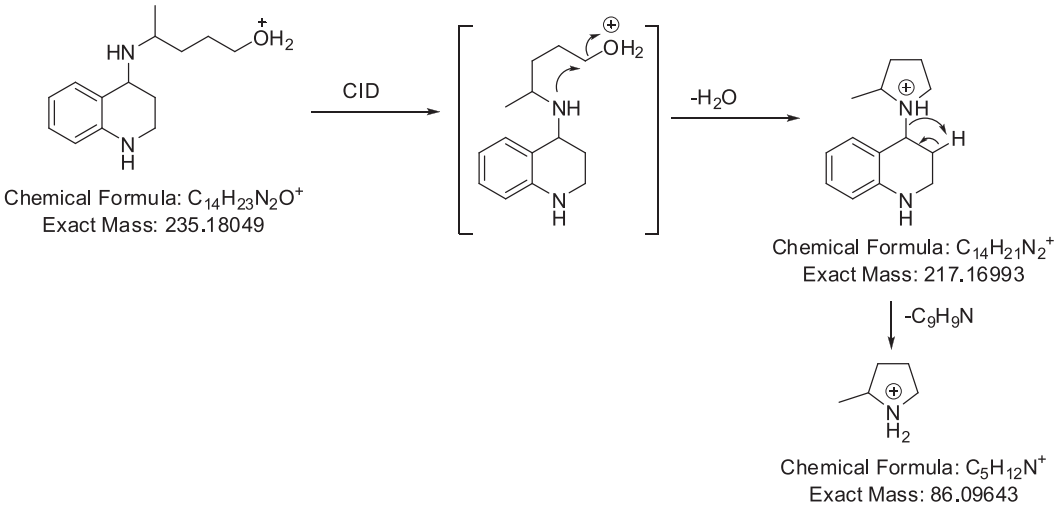

Fig. 4. Formation of the main fragment ions observed in the ESI-MS^2 spectra of HCQ-M.

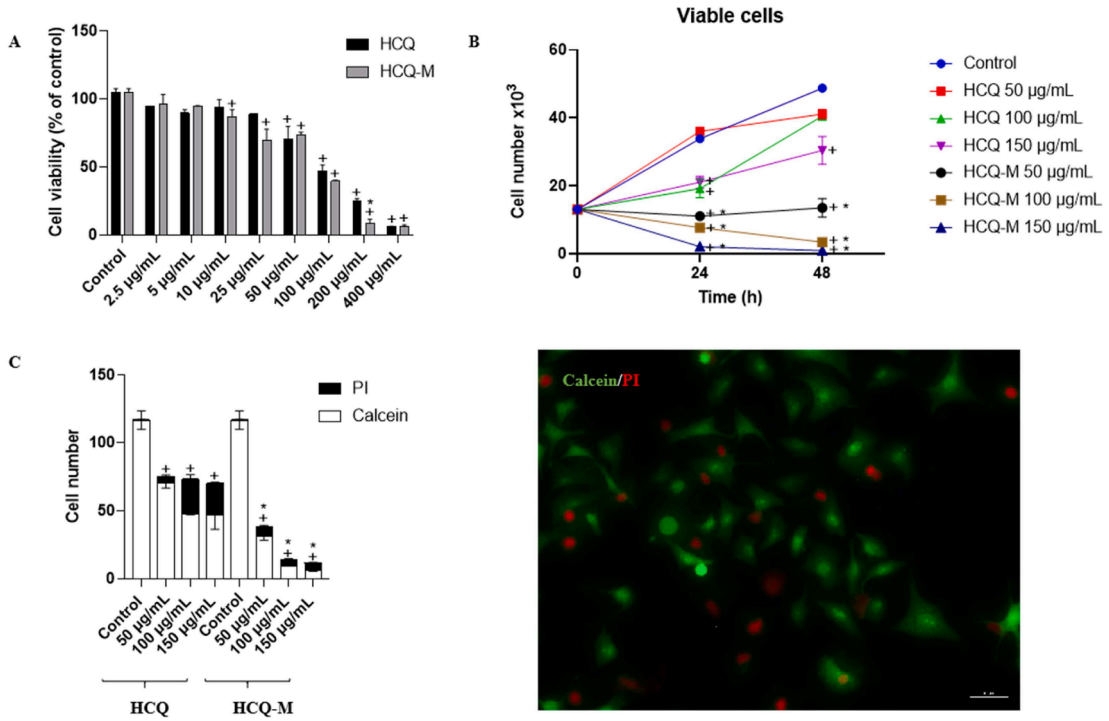

Fig. 5. Effect of hydroxychloroquine (HCQ) and its fungal metabolite (HCQ-M) on CCD-1059Sk cells. Cell viability was determined by (A) SRB assay; (B) Cell proliferation determined by trypan blue assay after 24 and 48 h; and (C) cell viability and apoptosis were analyzed by Calcein-AM and propidium iodide (PI) staining, respectively. The scale bar is 50 µm. (+) Significant differences from the control group (untreated) were determined according to the analysis of variance (ANOVA) followed by Tukey's test, and (*) significant differences between the HCQ and HCQ-M groups were determined according to the ANOVA followed by Bonferroni's post-test: $p < 0.05$. The data shown are mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

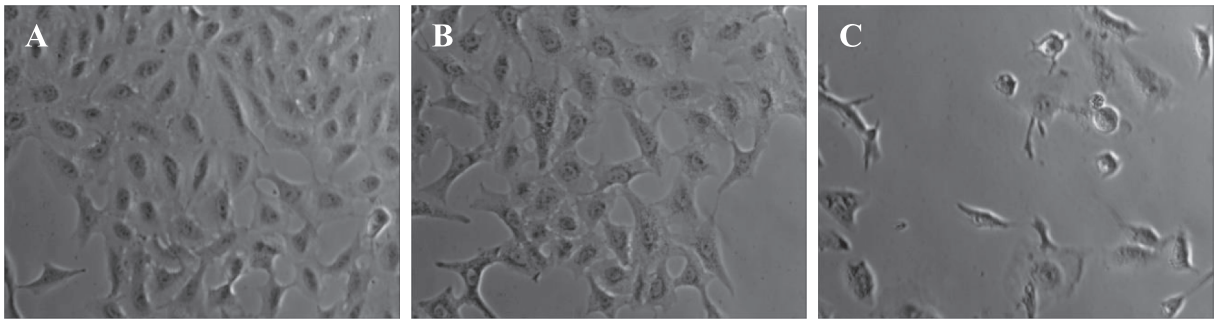

Fig. 6. Effect of hydroxychloroquine (HCQ) and its fungal metabolites (HCQ-M) on CCD-1059Sk cells. In optical microscopy, a representative image of control (A) and the change in cell morphology of treated cells with 50 µg/mL HCQ (B) and 50 µg/mL HCQ-M (C).

Conclusions

Human biotransformation of xenobiotics has classical steps classified in two phases (phase I and phase II) to inactivate and convert xenobiotics into more hydrophilic derivatives to be eliminated rapidly from the body. In summary, *C. echinulata* var. *elegans* was able to convert HCQ into a phase I metabolite through reactions similar to human metabolism. The herein-developed approach may provide compounds with related chemical structures in better yields when compared to *in vivo* studies to carry out biological and toxicological assays. Moreover, the biotransformation by *C. echinulata* var. *elegans* is an attractive alternative to reaching unknown derivatives of HCQ. The cytotoxicity evaluation performed with HCQ and its microbial metabolite (HCQ-M) showed higher toxicity of HCQ-M than HCQ to human fibroblasts. So, the discovery of more toxic HCQ metabolites will contribute to studying structure–activity relationships further and deep evaluation of HCQ safety.

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Author contributions

E.O.S. was responsible for the design of experiments, funding acquisition, investigation, and writing the paper. V.H.P.S. for the biotransformation assays. W.T.S, M.I., and A.C.C.P. for cytotoxic experiments and data analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

References

[1] S. Asha, M. Vidyavathi, Cunninghamella – a microbial model for drug metabolism studies – a review, Biotechnol. Adv. 27 (2009) 16–29, https://doi.org/10.1016/j.biotechadv.2008.07.005.

[2] M.A.D. Boaventura, R.F.A.P. Lopes, J.A. Takahashi, Microorganisms as tools in modern chemistry: the biotransformation of 3-indolylacetonitrile and tryptamine by fungi, Brazilian J. Microbiol. (2004) 345–347.

[3] P. Clark, Hydroxychloroquine compared with placebo in rheumatoid arthritis, Ann. Intern. Med. 119 (1993) 1067, https://doi.org/10.7326/0003-4819-119-11-199312010-00002.

[4] K. Faber, Biotransformations in Organic Chemistry: A Textbook, Springer-Verlag, Berlin, New York, 2004, p. 1953.

[5] K. Faber, Biotransformations, in: T. Scheper (Ed.), Advances in Biochemical Engineering / Biotechnologynology, Springer, Berlin Heidelberg, Berlin, 1999.

[6] P. Fasinu, P. J. Bouic, B. Rosenkranz, Liver-based in vitro technologies for drug biotransformation studies – a review, Curr. Drug Metab. 13 (2012) 215–224, https://doi.org/10.2174/138920012798918426.

[7] R.I. Fox, Mechanism of action of hydroxychloroquine as an antirheumatic drug, Semin. Arthritis Rheum. 23 (1993) 82–91, https://doi.org/10.1016/S0049-0172(10)80012-5.

[8] A.K. Ibrahim, M.M. Radwan, S.A. Ahmed, D. Slade, S.A. Ross, M.A. ElSohly, I. A. Khan, Microbial metabolism of cannflavin A and B isolated from Cannabis sativa, Phytochemistry 71 (2010) 1014–1019, https://doi.org/10.1016/j.phytochem.2010.02.011.

[9] S. Kebamo, S. Tesema, The role of biotransformation in drug discovery and development, J. Drug Metab. Toxicol. 06 (2015), https://doi.org/10.4172/2157-7609.1000196.

[10] S.L. Kelly, D.E. Kelly, Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? Philos. Trans. R. Soc. B Biol. Sci. 368 (2013) 20120476, https://doi.org/10.1098/rstb.2012.0476.

[11] W.U. Kim, S.A. Yoo, S.Y. Min, S.H. Park, H.S. Koh, S.W. Song, C.S. Cho, Hydroxychloroquine potentiates Fas-mediated apoptosis of rheumatoid synoviocytes, Clin. Exp. Immunol. 144 (2006) 503–511, https://doi.org/10.1111/j.1365-2249.2006.03070.x.

[12] O. Legrand, G. Simonin, J.Y. Perrot, R. Zittoun, J.P. Marie, Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients, Blood 91 (1998) 4480–4488.

[13] J. Liu, R. Cao, M. Xu, X. Wang, H. Zhang, H. Hu, Y. Li, Z. Hu, W. Zhong, M. Wang, Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro, Cell Discov. 6 (2020) 16, https://doi.org/10.1038/s41421-020-0156-0.

[14] E.W. McChesney, Animal toxicity and pharmacokinetics of hydroxychloroquine sulfate, Am. J. Med. 75 (1983) 11–18, https://doi.org/10.1016/0002-9343(83)91265-2.

[15] N.M. Njuguna, C. Masimirembwa, K. Chibale, Identification and characterization of reactive metabolites in natural products-driven drug discovery, J. Nat. Prod. 75 (2012) 507–513, https://doi.org/10.1021/np200786j.

[16] C.R. Paludo, E.A. da Silva-Junior, E. de Oliveira Silva, R. Vessecchi, N. Peporine Lopes, M. Tallarico Pupo, F. da Silva Emery, N. dos Santos Gonçalves, R. Alves dos Santos, N.A. Jacometti Cardoso Furtado, Inactivation of β-lapachone cytotoxicity by filamentous fungi that mimic the human blood metabolism, Eur. J. Drug Metab. Pharmacokinet. 42 (2017) 213–220, https://doi.org/10.1007/s13318-016-0337-2.

[17] D. Plantone, T. Koudriavtseva, Current and future use of chloroquine and hydroxychloroquine in infectious, immune, neoplastic, and neurological diseases: a mini-review, Clin. Drug Investig. 38 (2018) 653–671, https://doi.org/10.1007/s40261-018-0656-y.

[18] B. Ramser, A. Kokot, D. Metze, N. Weiß, T.A. Luger, M. Böhm, Hydroxychloroquine modulates metabolic activity and proliferation and induces autophagic cell death of human dermal fibroblasts, J. Invest. Dermatol. 129 (2009) 2419–2426, https://doi.org/10.1038/jid.2009.80.

[19] S.P. Rendic, Metabolism and interactions of Ivermectin with human cytochrome P450 enzymes and drug transporters, possible adverse and toxic effects, Arch. Toxicol. 95 (2021) 1535–1546, https://doi.org/10.1007/s00204-021-03025-z.

[20] Sibov, T.T., Aya, L., Miyaki, M., Mamani, J.B., Marti, L.C., Sardinha, L.R., Pavon, L. F., Oliveira, D.M. De, Cardenas, W.H., Gamarra, L.F., 2012. Avaliação da marcação de células-tronco mesenquimais de cordão umbilical com nanopartículas superparamagnéticas de óxido de ferro recobertas com Dextran e complexadas a Poli-L-Lisina. Einstein (São Paulo) [online] 10, 180–188.

[21] E.O. Silva, T.C. Carvalho, I.A. Parshikov, R.A. dos Santos, F.S. Emery, N.A.J. C. Furtado, Cytotoxicity of lapachol metabolites produced by probiotics, Lett. Appl. Microbiol. 59 (2014) 108–114, https://doi.org/10.1111/lam.12251.

[22] E.O. Silva, N.S. Goncalves, R.A. Dos Santos, N.A.J.C. Furtado, Microbial metabolism of atovaquone and cytotoxicity of the produced phase I metabolite, Eur. J. Drug Metab. Pharmacokinet. 41 (2016) 645–650, https://doi.org/10.1007/s13318-015-0294-1.

[23] Stokkermans, T.J., Goyal, A., Bansal, P., Trichonas, G., 2022. Chloroquine and hydroxychloroquine toxicity [WWW Document]. StatPearls. URL https://www.ncbi.nlm.nih.gov/books/NBK537086/ (accessed 2.14.22).

[24] S.-L. Tran, A. Puhar, M. Ngo-Camus, N. Ramarao, Trypan blue dye enters viable cells incubated with the pore-forming toxin HlyII of Bacillus cereus, PLoS One 6 (2011) e22876.

[25] V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening, Nat. Protoc. 1 (2006) 1112–1116.

[26] M.J. Vincent, E. Bergeron, S. Benjannet, B.R. Erickson, P.E. Rollin, T.G. Ksiazek, N. G. Seidah, S.T. Nichol, Chloroquine is a potent inhibitor of SARS coronavirus infection and spread, Virol. J. 2 (2005) 69, https://doi.org/10.1186/1743-422X-2-69.

[27] N.J. White, The treatment of malaria, N. Engl. J. Med. 335 (1996) 800–806, https://doi.org/10.1056/NEJM199609123351107.